PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/12, C12Q 1/68
C07K 15/00, G01N 33/50
A61K 39/00

A1

(11) International Publication Number: WO 92/03549

(43) International Publication Date: 5 March 1992 (05.03.92)

(21) International Application Number: PCT/NL91/00153

(22) International Filing Date: 15 August 1991 (15.08.91)

(30) Priority data:
9001832 16 August 1990 (16.08.90) NL

(71) Applicant *(for all designated States except US)*: EURO-DIAGNOSTICS B.V. [NL/NL]; Wilmersdorf 24, P.O. Box 2820, NL-7303 CG Apeldoorn (NL).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : ROOS, Maria, Helena [NL/NL]; Welgelegenlaan 22, NL-3971 HN Driebergen (NL).

(74) Agents: DE BRUIJN, Leendert, C. et al.; Nederlandsch Octrooibureau, Scheveningseweg 82, P.O. Box 29720, NL-2502 LS The Hague (NL).

(81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CI (OAPI patent), CM (OAPI patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, PL, RO, SD, SE (European patent), SN (OAPI patent), SU+,TD (OAPI patent), TG (OAPI patent), US.

Published
With international search report.

(54) Title: SPECIFIC DNA SEQUENCES OF A NEMATODE WHICH CAN BE USED FOR THE DIAGNOSIS OF INFECTION WITH THE NEMATODE

(57) Abstract

Method for the diagnosis of a worm infection, with which method DNA from the total DNA from the eggs of the nematode is amplified, in which method a DNA sequence comprising a species specific part of the 3′ half of the β-tubulin gene is used as primer or in which method two DNA sequences of parts of the β-tubulin gene are used which parts are conserved in various nematodes as primers, which primers are located in the DNA sequence of the β-tubulin gene in such a manner that the amplification product has species specific length per nematode. Preferably a method for the diagnosis of infection with *H. contortus* using a DNA sequence comprising a species specific part of the 3′ half of the β-tubulin gene of *H. contortus* as primer. The invention is also directed at a DNA sequence of a tubulin gene of *Haemonchus contortus* or a part thereof and preferably at a DNA sequence of the beta-tubulin gene of *Haemonchus contortus* or a part thereof. A method for the diagnosis of an infection with *Haemonchus contortus*, with which method the total DNA from the eggs of the nematode is applied to a (nylon) membrane and hybridised with a DNA probe being a sequence which comprises at least a part of one of the above mentioned sequences.

+ See back of page

Biochem. Cell Biol., 67; 770-789)].

Furthermore, it was apparent from previously published DNA-sequences of a number of nematodes (also of ß-tubulin, see Driscoll et al. The Journal of Biology, 1989, volume 109, pages 2993-3003) that the known nematode DNA sequences mostly resemble higher eucaryotic tubulin sequences. Various ß-tubulin genes occur in most higher eucaryotes. From what is presently known there appears to be more difference between the different types (genes) of ß-tubulin within one animal species than between the same type among different animal species. For example: the degree of conservation between tubulin type ß-4 and ß-5 of the human is 91,2 %, whereas conservation between type ß-4 of the human and type ß-4 of the chicken is 99,3 %.

Considering the state of the art as described above it is therefore surprising that DNA of ß-tubulin is species specific for H. contortus.

Fragments of a DNA-sequence according to the invention can be used as oligonucleotide probes for specifically determining H. contortus especially in mixed infections. Preferably the PCR method is used for this. Probes comprising a part of the sequence according to the invention react specifically with H. contortus, whereby a probe comprising a part of the ß-tubulin sequence is especially suited for use in specifically determining H. contortus in this manner.

Upon studying the conservation of DNA of tubulin genes it was found that the location of the introns is generally conserved. Introns generally occur in the 5' half of the tubulin gene. In the article of Driscoll et al. already cited it was demonstrated that introns also occur in the 3' half of the 3 ß-tubulin genes of the nematode C. elegans. These introns can be distinguished from the introns of the ß-tubulin genes of other higher eucaryotes.

It has now been found that the DNA-sequence of the 3' half of the ß-tubulin gene of H. contortus also contains introns. The 3' half of ß-tubulin of H. contortus has been found to contain a lot more introns than the 3' half of ß-tubulin of C. elegans and the introns also differ in length from the introns of C. elegans. The location and length of introns in the 3' half of the ß-tubulin gene appears not to be conserved in nematodes.

Further research of ß-tubulin genes of other nematodes further to the above mentioned discovery have revealed that there are other nematodes beside H. contortus and C. elegans which also contain non

conserved introns in the 3' half of their ß-tubulin genes.

The subject invention is therefore in general directed at the use of a DNA sequence of a specific nematode as oligonucleotide probe, which probe comprises a part of the DNA sequence of the 3' half of a ß-tubulin gene, for specifically determining the specific nematode. The subject invention is therefore not restricted to a method for specifically detecting H. contortus in which method a DNA sequence is used as oligonucleotide probe comprising a part of the 3' half of the ß-tubulin gene of H. contortus. The detection of H. contortus in this manner serves as an example of a method for the diagnosis of an infection with a certain nematode according to the subject invention.

Another suitable example of a nematode which can specifically be detected using an oligonucleotide probe comprising a part of the DNA sequence of the 3' half of the ß-tubulin gene is Trichostrongylus colubriformis.

The presence of differences in the 3' half of ß-tubulin genes of nematodes when compared with eachother made a species specific PCR possible. Parts of a DNA sequence comprising a ß-tubulin gene can be used as oligonucleotide probes for specifically detecting a nematode with the aid of the PCR in mixed infections.

A particular embodiment of the method that has been described above comprises the use of parts of the ß-tubulin gene as primers, which primers are conserved in various nematodes, whereby two sequences are selected as primers which are located in such a manner that the DNA sequence which is amplified from the total DNA of the nematode via PCR, the amplification product, has a species specific length per nematode, because the DNA sequence which is located between the primers is species specific. It is preferable to amplify DNA from the DNA sequence comprising a part of the 3' half of the ß-tubulin gene, as the DNA sequence of the 3' half has been found to differ in intron length per nematode.

Two ß-tubulin oligonucleotides, ß-1 and ß-2 were made on the basis of a DNA sequence of the ß-tubulin gene of H. contortus as determined by the inventors and illustrated in figure 2, in order to be able to measure the difference in introns among various nematode species. The oligonucleotides namely contain DNA sequences coding for amino acid sequences supposed to be conserved in ß-tubulin genes of all higher eucaryotes. Oligonucleotide ß-1 comprises amino acids 1-7 of the ß-tubulin gene of H. contortus and oligonucleotide ß-2 comprises amino

acids 198-204. The DNA sequence of ß-1 is: 5' ACGGATCTCAACCACCTTG 3' and the sequence of ß-2 on the complementary strand is 5' CTGGTACTGCTGGTATTCGGA 3'.

The PCR carried out with these oligonucleotides always gave a positive reaction to the presence of H. contortus DNA also in mixed infections. With this PCR a DNA fragment of approximately 1300 bases was generated. This PCR always gave a positive result when H. contortus DNA was present and then the fragment of 1300 bases was found with the ß-1 and ß-2 primers. The PCR always gave a negative reaction when no fragment of 1300 bases was present and no H. contortus DNA was present.

The PCR with the ß-1 and ß-2 primers was also tested on DNA of other prevalent sheep parasites. A positive reaction was also found with Trychostrongylus colubriformis also in mixed infections, however the DNA fragment was smaller approximately 900 bases and clearly discernable from the H. contortus fragment.

The PCR with the β-1 and β-2 primers was also tested on DNA of Ostertagia circumcincta, Nippostrongylus brasiliensis, Trichinella spiralis and Trichinella nativa. No reaction was found not even with so called degenerate primers, which take into account the degenaracy of the genetic code. These were primers β-7 (amino acids 1-6) with DNA sequence: 5' (CTA)G(GC)(GTAC)GA(CT)(CT)T(GCAT)AA(CT)CA(CT)(CT)T 3' and β-8 (amino acids 199-205) on the complementary strand with DNA sequence: 5' TC(CT)TGGTA(TC)TG(CT)TG(GA)TA(CT)TC 3' (figure 2). The PCR with the β-7 and β-8 primers was positive with DNA of H. contortus and T. colubriformis giving the DNA fragments that were described previously.

As the used primers correspond to a DNA sequence coding for a very conserved region of the β-tubulin molecule it appears probable that the β-tubulin gene(s) of the nematode species leading to a negative result with these primers using PCR contain an intron in the sequence of the primers. This could prevent the primer from binding and could prevent any reaction taking place. A change in the amino acid sequence in the region of the primers could also be a possible explanation for the above mentioned result but does not seem probable considering the conservation of the amino acid sequence in this region.

By selecting the primers in a different region of the 3' half of the β-tubulin gene a species specific PCR can also be obtained for the nematodes giving a negative result with the primers β-1, β-2, β-7 and β-8.

The subject invention is therefore also directed at a method

for the diagnosis of a worm infection, with which method the total DNA from the eggs from the nematode is amplified preferably via PCR, which method is characterized in the use of two parts of the coding sequence of the β-tubulin gene which are conserved in various nematodes as primers, which primers are located in the DNA sequence of the β-tubulin gene in such a manner that the amplification product has a species specific length per nematode.

The beta-tubulin probes comprising the complete 2,2 kb ß-tubulin clone of H. contortus (pEMBLaB, deposited at the CBS in Baarn under Nr. CBS 335.90) or the 1,3 kb ß-1/ß-2 PCR fragment always show a positive reaction to the presence of H. contortus, even in the case of a mixed infection. The beta-tubulin probes of H. contortus were also tested on the DNA of other frequently occurring sheep parasites and this reaction was found to be much less pronounced than with its own species. Yet specific bands were obtained with these large probes which indicates conservation. The weaker reaction could be caused by introns in conserved regions or small species specific DNA sequences within these large probes. The species which were tested are, inter alia: Cooperia circumcincta, Nematodirus battus, Nippostrongylus brasiliensis, Ostertagia circumcincta, Trichostrongylus colubriformis, Trichostrongylus vitrinus, Trichinella spiralis and Trichinella nativa.

By selecting a specific sequence from a DNA sequence according to the invention of for example, at least 10 nucleotides from a H. contortus specific intron or the DNA sequence coding for amino acids 332-335, which under specific conditions reacts solely with H. contortus, it is possible to establish a test by means of which it is possible, in the case of a mixed infection with nematodes, to diagnose infection with H. contortus from the DNA of the eggs.

Examples of tests which can be used are those which are used in the case of other nematodes:

a) the polymerase chain reaction (PCR), and

b) the dot-blot assay.

The present invention also relates to a method for the diagnosis of a worm infection, in which method the total DNA from the eggs from the nematode is applied to a nylon membrane and hybridised with a DNA probe, which method is characterised in that a diagnosis of infection with Haemonchus contortus is made, using a sequence which corresponds to a specific part of a tubulin gene of H. contortus as oligonucleotide probe. A preferred method is a method in which specific

DNA fragments from the DNA of the eggs are amplified, for example by means of PCR, and are rendered visible. The diagnosis of an infection with H. contortus can be made because the amplification of specific fragments is successful when H. contortus DNA is present in the egg sample and sequences are used, such as a primer which corresponds to at least a conserved part of a tubulin gene of H. contortus, whereby said sequences are localised in the DNA sequence of the β-tubulin gene in such a manner that the amplification product has a species specific length.

The invention is also directed at a kit for carrying out the diagnosis of a worm infection which kit comprises at least a species specific part of the 3' half of the β-tubulin gene of the nematode to be detected as oligonucleotide probe and is also provided with other means and materials which are apparent to an expert.

The invention is further directed at a kit for carrying out a method for the diagnosis of infection with H. contortus, which kit comprises at least a part of the DNA sequence according to the invention and is also provided with other means and materials apparent to an expert. Such a kit shall preferably comprise a part of the 3' half of the β-tubulin gene of H. contortus.

The invention is further directed at a kit for carrying a method for the diagnosis of infection with H. contortus, which kit comprises at least primers comprising coding DNA sequences of the β-tubulin gene which are conserved in various nematodes, which primers are located in such a manner in the DNA sequence of the β-tubulin gene of H. contortus that the amplification product has a species specific length. Such a kit shall preferably comprise primers giving a part of the 3' half of the β-tubulin gene of H. contortus as amplification product. Such a kit will comprise for example the β-1 and β-2 primers and/or the β-7 and β-8 primers. Furthermore such a kit is also provided with the means and materials apparent for an expert.

The sequences according to the invention can also be used if they are incorporated in a vector, an expression vector being preferred. The invention relates to vectors of this type and also to recombinant microorganisms which comprise a DNA sequence according to the invention.

The invention also relates to a peptide which comprises at least one fragment of the amino acid sequence which is coded by a DNA sequence according to the invention.

Peptides of, for example, at least 15 amino acids are suitable as antigen, for example for the generation of specific antiserum, or

monoclonal antibodies against H. contortus. The invention relates to these antibodies. Antibodies of this type can also be used for the diagnosis of an infection with Haemonchus contortus. The invention relates to this use and to the use of the antibodies in a kit for the diagnosis of infection with H. contortus.

EXAMPLE

Construction of the gene libraries of H. contortus and analysis of the tubulin clones

Genomic DNA was made from L3 larvae using the method of Coulson et al. (Coulson, A., Sulston, J., Brenner, S. and Karn, J. (1986) Toward a physical map of the genome of the nematode Caenorhabditis elegans, Proc. Natl. Acad. Sci. USA 83, 7821-7825). The size of the DNA fragments to be cloned was 20 kb. In order to obtain arbitrary fragments of this size a partial digestion was carried out with a restriction enzyme that cuts frequently in genomic DNA. To this end, the concentration of the enzyme was chosen as described in Maniatis et al. (Maniatis, T., Fritsch, T. and Sambrook, J. (1982) Molecular cloning. A laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., USA). These fragments were isolated and ligated in the DNA of the bacteriophage arms of EMBL3 using the method of Frischauf et al. (Frischauf, A.M., Lehrach, H., Poustka, A., Murray, N. (1983) Lambda replacement vectors carrying polylinker sequences, J. Mol. Biol. 170, 827-841). This DNA construct was then packaged in bacteriophage protein envelope and used to infect the bacterial strain Le 392. The infected bacteria were plated out with non-infected bacteria and after incubation at 37°C a plaque was visible at the location where infected bacteria were present (Frischauf, A.M., Lehrach, H., Poustka, A. and Murray, N. (1983) Lambda replacement vectors carrying polylinker sequences, J. Mol. Biol. 170, 827-841). The plaques were then screened according to the method of Maniatis et al. (Maniatis, T., Fritsch, T. and Sambrook, J. (1982) Molecular cloning. A laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., USA). The tubulin probes used to isolate the alpha-tubulin DNA-containing clones were chicken alpha-tubulin [as described in Valenzuela et al. (Valenzuela, P., Quiroga, M., Zaldivar, J., Rutter, W.J., Kirshner, M.W. and Cleveland, D.W. (1981) Nucleotide and corresponding amino acid sequences encoded by alpha and beta tubulin mRNAs. Nature 289, 650-655)] and Drosophila hydei alpha-tubulin [a gift from Dr. R. Brand of the Catholic University of Nijmegen (Michiels, F., Falkenburg, D., Muller, A.M., Hinz, U., Otto, U., Bellmann, R., Glazer, K.H., Brand, R.,

Bialojan, S. and Renkawitz-Pohl, R. (1987) Testis-specific beta2 tubulins are identical in Drosophila melanogaster and D. hydei but differ from the ubiquitous beta1 tubulin)].

The beta-tubulin probes used to isolate the beta-tubulin DNA-containing clones were a beta-tubulin clone of Caenorhabditis elegans, Ben-1 [which is described in Driscoll et al. (Driscoll, M., Dean, E., Reilly, E., Bergholz, E. and Chalfie, M. (1989) Genetic and molecular analysis of a Caenorhabditis elegans beta tubulin that conveys benzimidazole sensitivity, J. Cell Biol. 109, 2993-3003)] and a beta-tubulin clone of Drosophila hydei pTB5 [a gift from Dr. R. Brand of the Catholic University of Nijmegen (Michiels, F., Falkenburg, D., Muller, A.M., Hinz, U., Otto, U., Bellmann, R., Glazer, K.H., Brand, R., Bialojan, S. and Renkawitz-Pohl, R. (1987) Testis-specific beta2 tubulins are identical in Drosophila melanogaster and D. hydei but differ from the ubiquitous beta1 tubulin)].

Of the clones obtained in this way, the clone pEMBL8cA (deposited with the CBS in Baarn under No. CBS 335.90), for alpha-tubulin, and the clone pEMBL7aB (deposited with the CBS in Baarn under No. CBS 334.90), for beta-tubulin, were further characterised. Subclones thereof, which contain DNA fragments coding for protein, were constructed in the vectors Bluescript and M13. The DNA of these subclones was sequenced using the dideoxy method of Sanger et al. (Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain terminating inhibitors, Proc. Natl. Acad. Sci. USA 74, 5463-5467), as modified in the T7 sequencing kit from Pharmacia and the M13 sequencing kit from Amersham. Standard primers, which are supplied with the kits, were used. The analysis and comparison of the sequences thus obtained was carried out using the Microgenie (Beckman) and PC/Gene (Genofit, Geneva) computer programs. The sequences determined for these clones are shown in Figures 1 and 2 respectively.

The PCR specific for H. contortus and T. colubriformis.

The oligonucleotides ß-1 and ß-2 as described above were made in order to carry out a species specific PCR for DNA of H. contortus and T. colubriformis. The ß-7 and ß-8 primers were made in order to obtain a species specific PCR for the species O. circumcincta, T. spiralis and T. nativa. The PCR method was used according to Ehrlich et al. (Saiki, R.K., (1989) in: PCR technology, principles and applications for DNA amplification, Erlich, H.A. (ed.), Stockton Press, New York, London, pp. 7-16). The reaction conditions used in the present invention, which were

used to amplify the DNA, are: PCR with ß-1 and ß-2 primers with a magnesium concentration of 1,5 mM an annealing temperature of 61°C for 30 seconds, elongation at 72°C for 2 minutes and denaturing at 95°C for 30 seconds. This method amplifies the beta-tubulin of H. contortus and T. colubriformis from the genomic DNA, which can be rendered visible by electrophoresis in an agarose gel, whereby DNA of H. contortus gives a visible band of 1,3 kb and DNA of T. colubriformis gives a visible band of 0,9 kb. This method does not work with Ostertagia, N. brasiliensis, T. spiralis and T. nativa, which makes it clear that this PCR method with the abovementioned primers is specific for Haemonchus contortus and Trichostrongylus colubriformis.

The PCR conditions with the β-7 and β-8 primers were: 5 cycli as follows. An annealing temperature of 37°C for 1 minute, a slow rise in 5 minutes from 37°C to the elongation temperature of 72°C, 2 minutes at 72°C, 1 minute at 95°C. Subsequently 20 cycli of 1 minute at 55°C, 2 minutes at 72°C and 1 minute at 95°C. The magnesium concentration was 1,5 mM. This PCR visualized a positive reaction with H. contortus DNA and was negative with O. circumcincta, T. spiralis and T. nativa.

CLAIMS

1. DNA sequence of a tubulin gene of Haemonchus contortus or a part thereof.

2. DNA sequence of the beta-tubulin gene of Haemonchus contortus or a part thereof.

3. DNA sequence according to Claim 2, characterised in that this comprises the nucleotide sequence according to Fig. 2 or a part thereof.

4. DNA sequence according to Claim 3, characterised in that this is obtainable from clone pEMBL7aB (deposited with the CBS in Baarn under No. CBS 334.90).

5. DNA sequence according to Claim 1, characterised in that this comprises the nucleotide sequence according to Fig. 1 or a part thereof.

6. DNA sequence according to Claim 5, characterised in that this is obtainable from clone pEMBL8cA (deposited with the CBS in Baarn under No. CBS 335.90).

7. Method for the diagnosis of a worm infection, with which method the total DNA from the eggs of the nematode is applied to a (nylon) membrane and hybridised with a DNA probe, characterised in that a diagnosis of infection with Haemonchus contortus is made, the DNA probe used being a species specific sequence which comprises at least a part of a sequence according to one of the preceding claims.

8. Method for the diagnosis of a worm infection, with which method DNA from the total DNA from the eggs of the nematode is amplified, characterised in that a diagnosis of infection with Haemonchus contortus is made the primers used being DNA sequences which comprise at least a part of a DNA sequence according to one of Claims 1-6.

9. Method for the diagnosis of a worm infection, with which method DNA from the total DNA from the eggs of the nematode is amplified, characterised in that DNA sequences comprising species specific parts of the 3' half of the β-tubulin gene are used as primers.

10. Method for the diagnosis of a worm infection according to claim 9, characterised in that the diagnosis of infection with H. contortus is carried out using DNA sequences comprising species specific parts of the 3' half of the β-tubulin gene of H. contortus as primers.

11. Method for the diagnosis of a worm infection, with which method DNA from the total DNA from the eggs of the nematode is amplified, characterised in that two DNA sequences of the β-tubulin gene are used

monoclonal antibodies against H. contortus. The invention relates to these antibodies. Antibodies of this type can also be used for the diagnosis of an infection with Haemonchus contortus. The invention relates to this use and to the use of the antibodies in a kit for the diagnosis of infection with H. contortus.

EXAMPLE

Construction of the gene libraries of H. contortus and analysis of the tubulin clones

Genomic DNA was made from L3 larvae using the method of Coulson et al. (Coulson, A., Sulston, J., Brenner, S. and Karn, J. (1986) Toward a physical map of the genome of the nematode Caenorhabditis elegans, Proc. Natl. Acad. Sci. USA 83, 7821-7825). The size of the DNA fragments to be cloned was 20 kb. In order to obtain arbitrary fragments of this size a partial digestion was carried out with a restriction enzyme that cuts frequently in genomic DNA. To this end, the concentration of the enzyme was chosen as described in Maniatis et al. (Maniatis, T., Fritsch, T. and Sambrook, J. (1982) Molecular cloning. A laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., USA). These fragments were isolated and ligated in the DNA of the bacteriophage arms of EMBL3 using the method of Frischauf et al. (Frischauf, A.M., Lehrach, H., Poustka, A., Murray, N. (1983) Lambda replacement vectors carrying polylinker sequences, J. Mol. Biol. 170, 827-841). This DNA construct was then packaged in bacteriophage protein envelope and used to infect the bacterial strain Le 392. The infected bacteria were plated out with non-infected bacteria and after incubation at 37°C a plaque was visible at the location where infected bacteria were present (Frischauf, A.M., Lehrach, H., Poustka, A. and Murray, N. (1983) Lambda replacement vectors carrying polylinker sequences, J. Mol. Biol. 170, 827-841). The plaques were then screened according to the method of Maniatis et al. (Maniatis, T., Fritsch, T. and Sambrook, J. (1982) Molecular cloning. A laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., USA). The tubulin probes used to isolate the alpha-tubulin DNA-containing clones were chicken alpha-tubulin [as described in Valenzuela et al. (Valenzuela, P., Quiroga, M., Zaldivar, J., Rutter, W.J., Kirshner, M.W. and Cleveland, D.W. (1981) Nucleotide and corresponding amino acid sequences encoded by alpha and beta tubulin mRNAs. Nature 289, 650-655)] and Drosophila hydei alpha-tubulin [a gift from Dr. R. Brand of the Catholic University of Nijmegen (Michiels, F., Falkenburg, D., Muller, A.M., Hinz, U., Otto, U., Bellmann, R., Glazer, K.H., Brand, R.,

Bialojan, S. and Renkawitz-Pohl, R. (1987) Testis-specific beta2 tubulins are identical in Drosophila melanogaster and D. hydei but differ from the ubiquitous beta1 tubulin)].

The beta-tubulin probes used to isolate the beta-tubulin DNA-containing clones were a beta-tubulin clone of Caenorhabditis elegans, Ben-1 [which is described in Driscoll et al. (Driscoll, M., Dean, E., Reilly, E., Bergholz, E. and Chalfie, M. (1989) Genetic and molecular analysis of a Caenorhabditis elegans beta tubulin that conveys benzimidazole sensitivity, J. Cell Biol. 109, 2993-3003)] and a beta-tubulin clone of Drosophila hydei pTB5 [a gift from Dr. R. Brand of the Catholic University of Nijmegen (Michiels, F., Falkenburg, D., Muller, A.M., Hinz, U., Otto, U., Bellmann, R., Glazer, K.H., Brand, R., Bialojan, S. and Renkawitz-Pohl, R. (1987) Testis-specific beta2 tubulins are identical in Drosophila melanogaster and D. hydei but differ from the ubiquitous beta1 tubulin)].

Of the clones obtained in this way, the clone pEMBL8cA (deposited with the CBS in Baarn under No. CBS 335.90), for alpha-tubulin, and the clone pEMBL7aB (deposited with the CBS in Baarn under No. CBS 334.90), for beta-tubulin, were further characterised. Subclones thereof, which contain DNA fragments coding for protein, were constructed in the vectors Bluescript and M13. The DNA of these subclones was sequenced using the dideoxy method of Sanger et al. (Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain terminating inhibitors, Proc. Natl. Acad. Sci. USA 74, 5463-5467), as modified in the T7 sequencing kit from Pharmacia and the M13 sequencing kit from Amersham. Standard primers, which are supplied with the kits, were used. The analysis and comparison of the sequences thus obtained was carried out using the Microgenie (Beckman) and PC/Gene (Genofit, Geneva) computer programs. The sequences determined for these clones are shown in Figures 1 and 2 respectively.

The PCR specific for H. contortus and T. colubriformis.

The oligonucleotides ß-1 and ß-2 as described above were made in order to carry out a species specific PCR for DNA of H. contortus and T. colubriformis. The ß-7 and ß-8 primers were made in order to obtain a species specific PCR for the species O. circumcincta, T. spiralis and T. nativa. The PCR method was used according to Ehrlich et al. (Saiki, R.K., (1989) in: PCR technology, principles and applications for DNA amplification, Erlich, H.A. (ed.), Stockton Press, New York, London, pp. 7-16). The reaction conditions used in the present invention, which were

used to amplify the DNA, are: PCR with ß-1 and ß-2 primers with a magnesium concentration of 1,5 mM an annealing temperature of 61°C for 30 seconds, elongation at 72°C for 2 minutes and denaturing at 95°C for 30 seconds. This method amplifies the beta-tubulin of H. contortus and T. colubriformis from the genomic DNA, which can be rendered visible by electrophoresis in an agarose gel, whereby DNA of H. contortus gives a visible band of 1,3 kb and DNA of T. colubriformis gives a visible band of 0,9 kb. This method does not work with Ostertagia, N. brasiliensis, T. spiralis and T. nativa, which makes it clear that this PCR method with the abovementioned primers is specific for Haemonchus contortus and Trichostrongylus colubriformis.

The PCR conditions with the β-7 and β-8 primers were: 5 cycli as follows. An annealing temperature of 37°C for 1 minute, a slow rise in 5 minutes from 37°C to the elongation temperature of 72°C, 2 minutes at 72°C, 1 minute at 95°C. Subsequently 20 cycli of 1 minute at 55°C, 2 minutes at 72°C and 1 minute at 95°C. The magnesium concentration was 1,5 mM. This PCR visualized a positive reaction with H. contortus DNA and was negative with O. circumcincta, T. spiralis and T. nativa.

CLAIMS

1. DNA sequence of a tubulin gene of Haemonchus contortus or a part thereof.

2. DNA sequence of the beta-tubulin gene of Haemonchus contortus or a part thereof.

3. DNA sequence according to Claim 2, characterised in that this comprises the nucleotide sequence according to Fig. 2 or a part thereof.

4. DNA sequence according to Claim 3, characterised in that this is obtainable from clone pEMBL7aB (deposited with the CBS in Baarn under No. CBS 334.90).

5. DNA sequence according to Claim 1, characterised in that this comprises the nucleotide sequence according to Fig. 1 or a part thereof.

6. DNA sequence according to Claim 5, characterised in that this is obtainable from clone pEMBL8cA (deposited with the CBS in Baarn under No. CBS 335.90).

7. Method for the diagnosis of a worm infection, with which method the total DNA from the eggs of the nematode is applied to a (nylon) membrane and hybridised with a DNA probe, characterised in that a diagnosis of infection with Haemonchus contortus is made, the DNA probe used being a species specific sequence which comprises at least a part of a sequence according to one of the preceding claims.

8. Method for the diagnosis of a worm infection, with which method DNA from the total DNA from the eggs of the nematode is amplified, characterised in that a diagnosis of infection with Haemonchus contortus is made the primers used being DNA sequences which comprise at least a part of a DNA sequence according to one of Claims 1-6.

9. Method for the diagnosis of a worm infection, with which method DNA from the total DNA from the eggs of the nematode is amplified, characterised in that DNA sequences comprising species specific parts of the 3' half of the β-tubulin gene are used as primers.

10. Method for the diagnosis of a worm infection according to claim 9, characterised in that the diagnosis of infection with H. contortus is carried out using DNA sequences comprising species specific parts of the 3' half of the β-tubulin gene of H. contortus as primers.

11. Method for the diagnosis of a worm infection, with which method DNA from the total DNA from the eggs of the nematode is amplified, characterised in that two DNA sequences of the β-tubulin gene are used

which are conserved in various nematodes as primers, which primers are located in the DNA sequence of the β-tubulin gene in such a manner that the amplification product has species specific length per nematode.

12. A method according to claim 11, characterised in that β-1 and β-2 are used as primers, whereby β-1 has the sequence 5' ACGGATCTCAACCACCTTG 3' and β-2 has the sequence 5' CTGGTACTGCTGGTATTCGGA 3' on the complementary strand, or primers β-7 and β-8 are used whereby β-7 comprises the amino acids 1-6 of β-tubulin of H. contortus with DNA sequence: 5' (CTA)G(GC)(GTAC)GA(CT)(CT)T(GCAT)AA(CT)CA(CT)(CT)T 3' and β-8 comprises the amino acids 199-205 of β-tubulin of H. contortus with DNA sequence : 5' TC(CT)TGGTA(TC)TG(CT)TG(GA)TA(CT)TC 3' on the complementary strand (figure 2).

13. A method according to claim 12, characterised in that a diagnosis of infection is carried out which is specific for H. contortus when the amplification product has a length of 1,3 kb.

14. A method according to claim 12, characterised in that a diagnosis of infection if carried out which is specific for Trichostrongylus colubriformis when the amplification product has a length of 0,9 kb.

15. Peptide which comprises at least one fragment of the amino acid sequence which is encoded by a DNA sequence according to one of Claims 1-6.

16. Vector which comprises at least one DNA sequence according to one of Claims 1-6.

17. Vector according to Claim 16, characterised in that this is an expression vector which can express the DNA sequence according to one of Claims 1-6.

18. Recombinant microorganism which comprises a DNA sequence according to one of Claims 1-6 or a vector according to Claim 16 or 17.

19. Antibody which is generated against a peptide according to Claim 15.

20. Use of an antibody according to Claim 19 for diagnosing an infection with Haemonchus contortus.

21. Vaccine for protection against or for combating Haemonchus contortus, which comprises a peptide according to Claim 15 or a vector according to Claim 17 or a recombinant microorganism according to Claim 18.

22. Vaccine for combating Haemonchus contortus, which comprises an antibody according to Claim 19.

23. Kit for carrying out a diagnosis of infection with Haemonchus contortus, which comprises an antibody according to Claim 19.

24. Kit for carrying out a diagnosis of a worm infection which kit comprises oligonucleotide probes comprising a species specific part of the 3' half of the β-tubulin gene of the nematode to be detected.

25. Kit for carrying out the diagnosis of infection with H. contortus, which kit comprises at least a part of the DNA sequence according to one of the claims 1-6.

26. Kit for carrying out a diagnosis of infection with H. contortus, which kit comprises at least primers comprising coding DNA sequences of the β-tubulin gene which are conserved in various nematodes, which primers are located in such a manner in the DNA sequence of the β-tubulin gene of H. contortus that the amplification product has a species specific length.

27. Kit according to claim 25 or 26, which kit comprises a part of the DNA sequence of the 3' half of the β-tubulin gene of H. contortus.

28. Kit according to claim 27 which kit comprises the primers β-1 and β-2 and/or the primers β-7 and β-8.

Fig. 1

```
        10          20          30          40          50
TTTGCATGCT GTCCAACACG ACGCGATCGC TGAAGCTTGG GCTCGTTTGG ATATAAG

 60          70          80          90          100
TTT GAC CTT ATG TAT GCC AAG CGT GCA TTC GTC CAC TG GTGAG
Phe Asp Leu Met Tyr Ala Lys Arg Ala Phe Val His Trp
                                    10

      110         120         130         140         150
TGTTCTTTCG ACATCATCTT TTTCATTTGC AGTTGTTCTG CATATACAAC

      160         170         180          190          200
ATTTTATGAA AGTCAGATAT ACTGTTCAG G TAT GTC GGA GAG GGA ATG GAG
                                  Tyr Val Gly Glu Gly Met Glu
                                                          20

       210          220         230         240         250
GAA GGA GAG TTC AGT GAG GNA C GTGAAGA TCTCCCCGGG CTGCAGGAAT
Glu Gly Glu Phe Ser Glu

      260         270
TCGATATCAA GCTTATCGAT ACCGT
```

Fig. 2 (1)

primer β-7

--- --- --- --- --- ---

primer β-1

--- --- --- --- --- --- -

```
                                                                    50
ACG GAT CTC AAC CAC CTT G G TAATTGTTAT TACACTTACT AAAGTATACT
Thr Asp Leu Asn His Leu

                                                                   100
TAGACCTTTT TTCATGCTGA AAATGTGCAA TTGAAG TG TCT GTC ACA ATG TCT
                            Val Ser Val Thr Met Ser
                             10

                                                        140
GGT GTC ACG ACC TGC CTT CGA TTC CCT GGA CAG CTG AAT GCT GAT
Gly Val Thr Thr Cys Leu Arg Phe Pro Gly Gln Leu Asn Ala Asp
                            20

                                                              190
CTT CGC AAG TTA GCC GTG AAC ATG GTT CCA TTC CCT CGT CTT CAC
Leu Arg Lys Leu Ala Val Asn Met Val Pro Phe Pro Arg Leu His
        30                                          40

                                                        230
TTC TTC ATG CCC GGT TTT GCT CCA CTG TCT GCA AAG GGT GCT CAA
Phe Phe Met Pro Gly Phe Ala Pro Leu Ser Ala Lys Gly Ala Gln
                            50

                                                              280
GCA TAT CGC GCT TCG ACA GTT GCT GAG CTT ACA CAG CAA GTA
Ala Tyr Arg Ala Ser Thr Val Ala Glu Leu Thr Gln Gln
        60                                          70
```

2(2)

330

CGCCCATTAC TCTTTATCAG CATCAGTGAT TCAAAATTAC CAGCCAGCGC ATTTAG

380

ATG TTC GAT GCA AAG AAC ATG ATG GCT GCC TGT GAT CCT CGC CAT
Met Phe Asp Ala Lys Asn Met Met Ala Ala Cys Cys Pro Arg His
80

420

GGA CGT TAT CTT ACG GTC GCT GCT ATG TTC CGT GGT CGT ATG AGC
Gly Arg Tyr Leu Thr Val Ala Ala Met Phe Arg Gly Arg Asn Ser
90 100

480

ATG CGA GTGAGTAT TTTTAAACGT GTTTTTTCAT CAAGTAAATG ATGAATACTG
Met Arg

530

GTGGGACTGG AAATGAGTCA GAAGTAGTGT TCTAATGTGA GCTATTCACA

580

AAAACTTGTA TCTCACTGTG GGCCAAGACT TCTACATGGC AAGCCAGATG

630

TTTTATAAAT ACAGTGCTAT GAGTAGCAAC CATATATAGT TTCTATAAGA

680

TCGCTCTAAG ATATGAATGC CACCGGTATA ACTAGTTACT GGTTACTTAT

730

GCCATGAAGG AGTAGTAATT GATGCAGGAT GTCATCTGCT ATAATCTAAT

780

CGCAATAATA TTGTAGCTTT GAGAGACCTG CAGCATCCTC GCTACCGTAG

830

CCCGCATAGT TTTTTAAGCG AAACGGGTAG GCTATTGTGG TTTTTTGGGG

2(3)

880

GCATTTTTTT CTGAAATGCT GCAGTCAATT GTAAAACCGC GATGTGATTG

930

TGTGATCGGT TCATTGCAGT ATTTTTGATC GTAGAGATAA GAAAATATCA

980

TGTGGAAGGT CAACTGCTTC ATTTCAG GAA GTA GAT GAT CAG ATG ATG TCC
Glu Val Asp Asp Gln Met Met Ser
110

1020

GTG CAG AAC AAG AAC TCA TCA TAT TTC GTG GAA TGG ATT CCA AAC
Val Gln Asn Lys Asn Ser Ser Tyr Phe Val Glu Trp Ile Pro Asn
120

1070

AAC GTT AAG ACT GCC GTT TGT GAC ATT CCT CCT CGT GGA CTG AAA
Asn Val Lys Thr Ala Val Cys Asp Ile Pro Pro Arg Gly Leu Lys
130 140

1110

ATG GCG GCT ACC TTC GTT GGT AAC TCG ACT GCT ATC CAG GAG CTG
Met Ala Ala Thr Phe Val Gly Asn Ser Thr Ala Ile Gln Glu Leu
150

1160

TTC AAG CGT ATT TCG GAG CAA TTC ACT G GTTTGT TTTGATTTAT
Phe Lys Arg Ile Ser Glu Gln Phe Thr
160

1210

GAGCTGTCGT ACATGAGTGC GTCTTGATTC CAACACTCTT CACCGCTTCA G CC
Ala

1250

ATG TTC CGA CGC AAA GCT TTC CTT CAT TGG TAC ACT GGT GAG GGT
Met Phe Arg Arg Lys Ala Phe Leu His Trp Tyr Thr Gly Glu Gly
170 180

2(4)

1300

ATG GAC GAA ATG GAG TTC ACA GAA GCT GAG TCG AAC ATG AAT GAC

Met Asp Glu Met Glu Phe Thr Glu Ala Glu Ser Asn Met Asn Asp

190

primer β-8

--- --- --- --- --- --- --

primer β-2

--- --- --- --- --- --- ---

1350

CTT ATC TCC GAA TAC CAG CAG TAC CAG GTCCGTATTA TCTTATTCCA

Leu Ile Ser Glu Tyr Gln Gln Tyr Gln

200

1400

GCTATGTCTT AGCGTGATCG TAGACGTTTA GTCTTTAATA TTTCCAG GAA GCT

Glu Ala

1440

ACC GCT GAC GAT ATG GGC GAT CTC GAT GCA GAA GGT GGA GAA GAG

Thr Ala Asp Asp Met Gly Asp Leu Asp Ala Glu Gly Gly Glu Glu

210 220

1500

GCA TAT CCC GAG GAG TAA TGAT CCACAAAGTT GTGCTCTTTT TCCTGTGTCA

Ala Tyr Pro Glu Glu End

226

primer β-4

- ---------- ---------

1550

ATGCGAAATA CACATTGGTT GCGTTGTGTA TGGTGTTATT AAAGCTTGCT

2(4)

1300

ATG GAC GAA ATG GAG TTC ACA GAA GCT GAG TCG AAC ATG AAT GAC

Met Asp Glu Met Glu Phe Thr Glu Ala Glu Ser Asn Met Asn Asp

190

primer β-8

--- --- --- --- --- --- --

primer β-2

--- --- --- --- --- --- ---

1350

CTT ATC TCC GAA TAC CAG CAG TAC CAG GTCCGTATTA TCTTATTCCA

Leu Ile Ser Glu Tyr Gln Gln Tyr Gln

200

1400

GCTATGTCTT AGCGTGATCG TAGACGTTTA GTCTTTAATA TTTCCAG GAA GCT

Glu Ala

1440

ACC GCT GAC GAT ATG GGC GAT CTC GAT GCA GAA GGT GGA GAA GAG

Thr Ala Asp Asp Met Gly Asp Leu Asp Ala Glu Gly Gly Glu Glu

210 220

1500

GCA TAT CCC GAG GAG TAA TGAT CCACAAAGTT GTGCTCTTTT TCCTGTGTCA

Ala Tyr Pro Glu Glu End

226

primer β-4

- ---------- ---------

1550

ATGCGAAATA CACATTGGTT GCGTTGTGTA TGGTGTTATT AAAGCTTGCT

INTERNATIONAL SEARCH REPORT

International Application No PCT/NL 91/00153

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 12 N 15/12 C 12 Q 1/68 C 07 K 15/00
G 01 N 33/50 A 61 K 39/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 12 N C 12 Q C 07 K A 61 K G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	Parasitology Today, volume 6, no. 4, April 1990, M.H. Roos: "The molecular nature of benzimidazole resistance in Helminths", page 125, see page 125, column 1, line 48 - column 2, line 33; page 126, column 3, lines 44-63 ---	1-8
Y	The Journal of Cell Biology, volume 109, no. 6, December 1989, The Rockefeller University Press, M. Driscoll et al.: "Genetic and molecular analysis of a caenorhabditis elegans beta-tubulin that conveys benzimidazole sensitivity", pages 2993-3003, see the whole article ---	1-8
A	International Journal for Parasitology, volume 17, no. 3, 1987, Pergamon Journals Ltd (GB), E. Lacey et al.: "Further investigations of the primary mechanism of benzimidazole resistance in haemonchus contortus", pages 1421-1429 --- -/-	

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
06-11-1991	02. 12. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Nicole De Bie

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	Molecular and Biological Parasitology, volume 43, 4 October 1990, Elsevier Science Publishers B.V., M.H. Roos et al.: "Molecular analysis of selection for benzimidazole resistance in the sheep parasite haemonchus contortus", pages 77-88, see the whole article	1-8